Effect of EGF-receptor tyrosine kinase inhibitor on Rab5 function during endocytosis.

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Running title: vesicle membrane fusion is regulated by receptor tyrosine kinase activity

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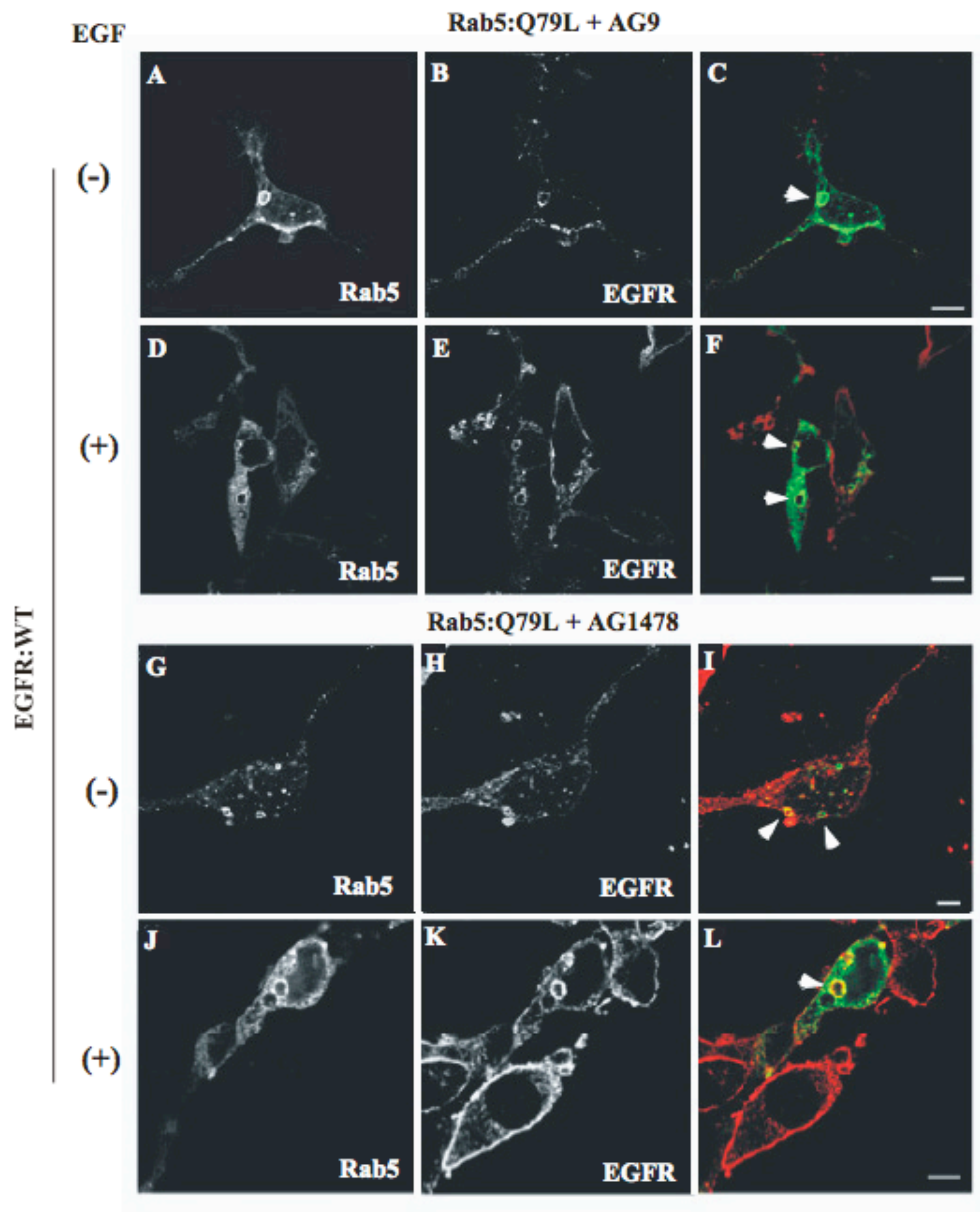
Rab5:Q79L + AG9
EGF
A
B
C
(-)
Rab5
EGFR
D
E
F
(+)
Rab5
EGFR
Rab5:Q79L + AG1478
EGFR:WT
G
H
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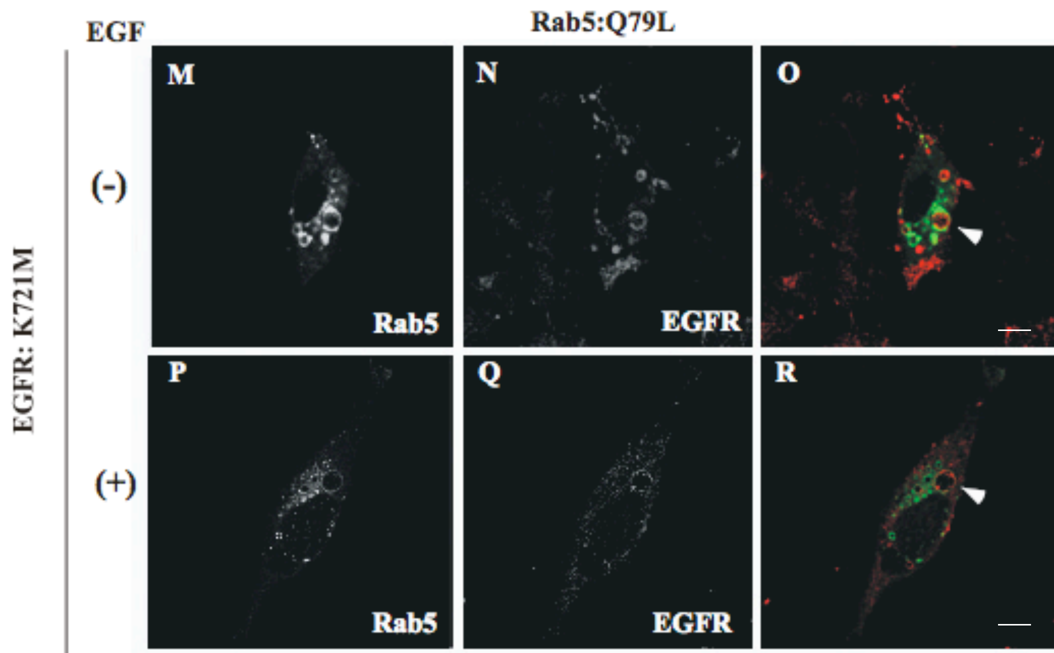
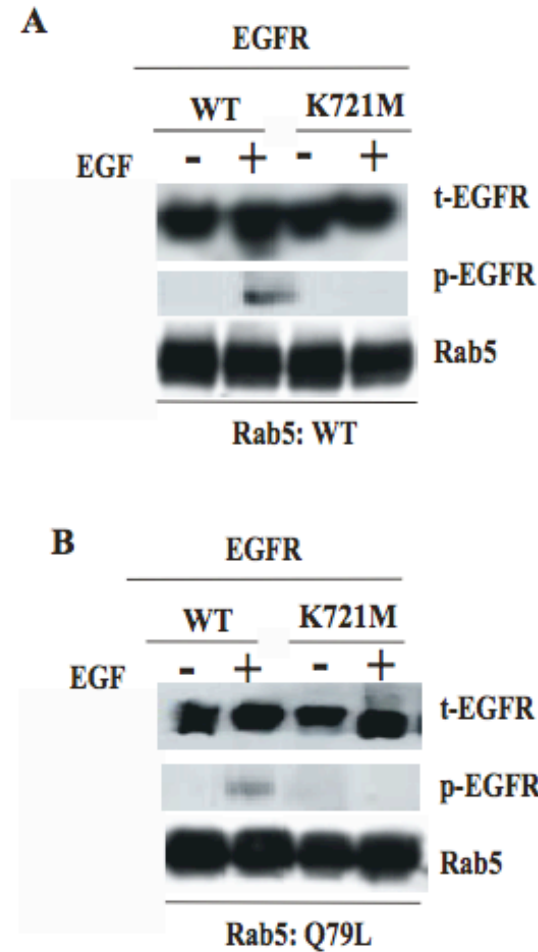
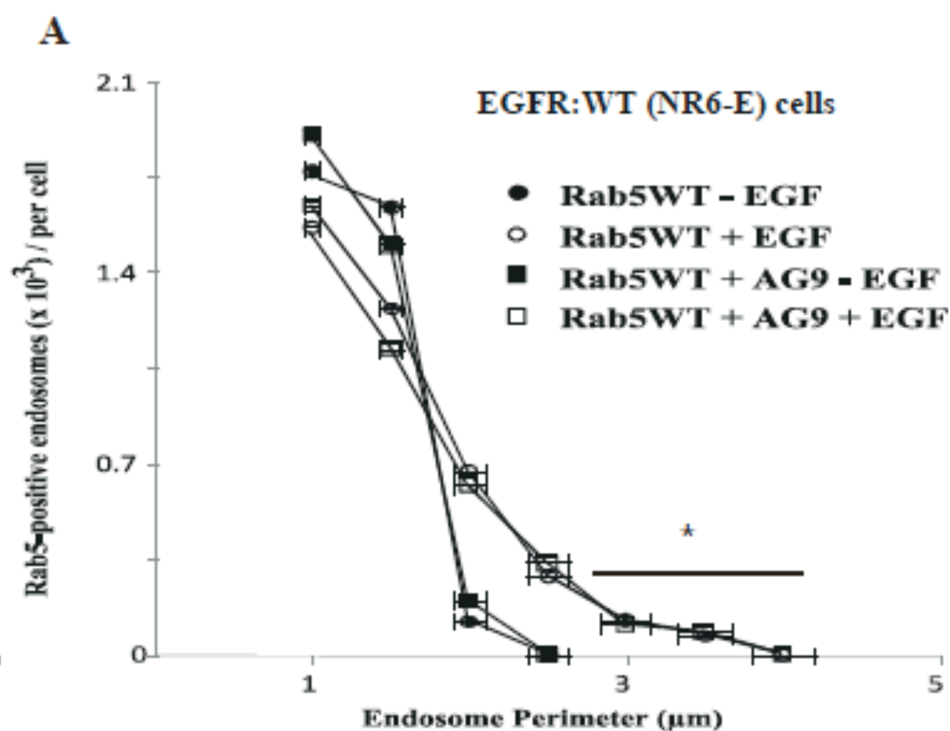
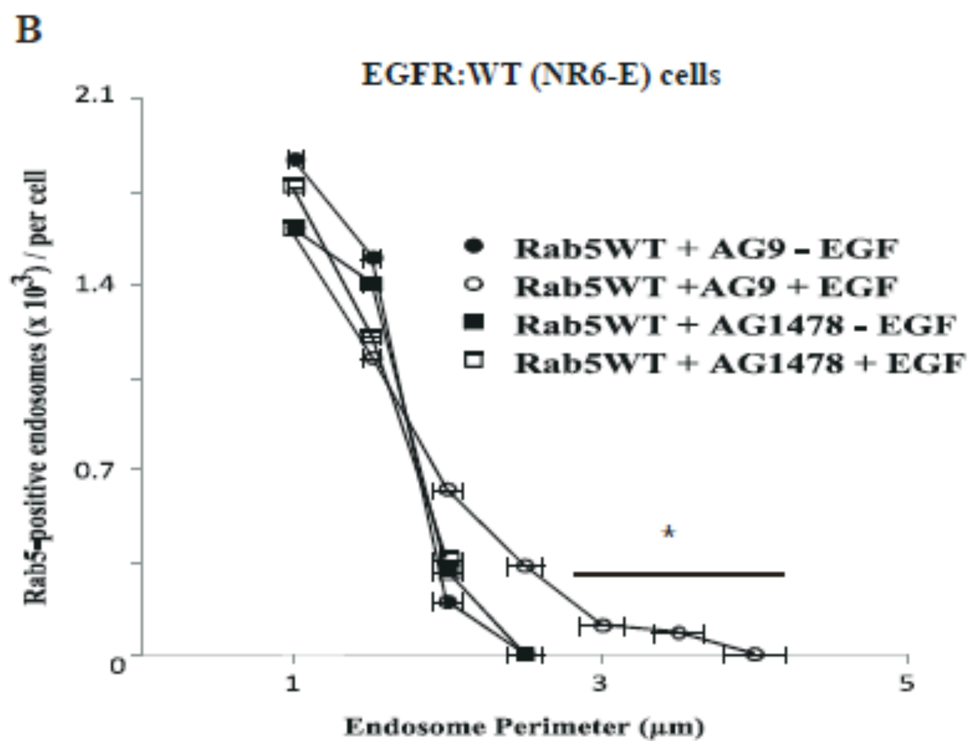


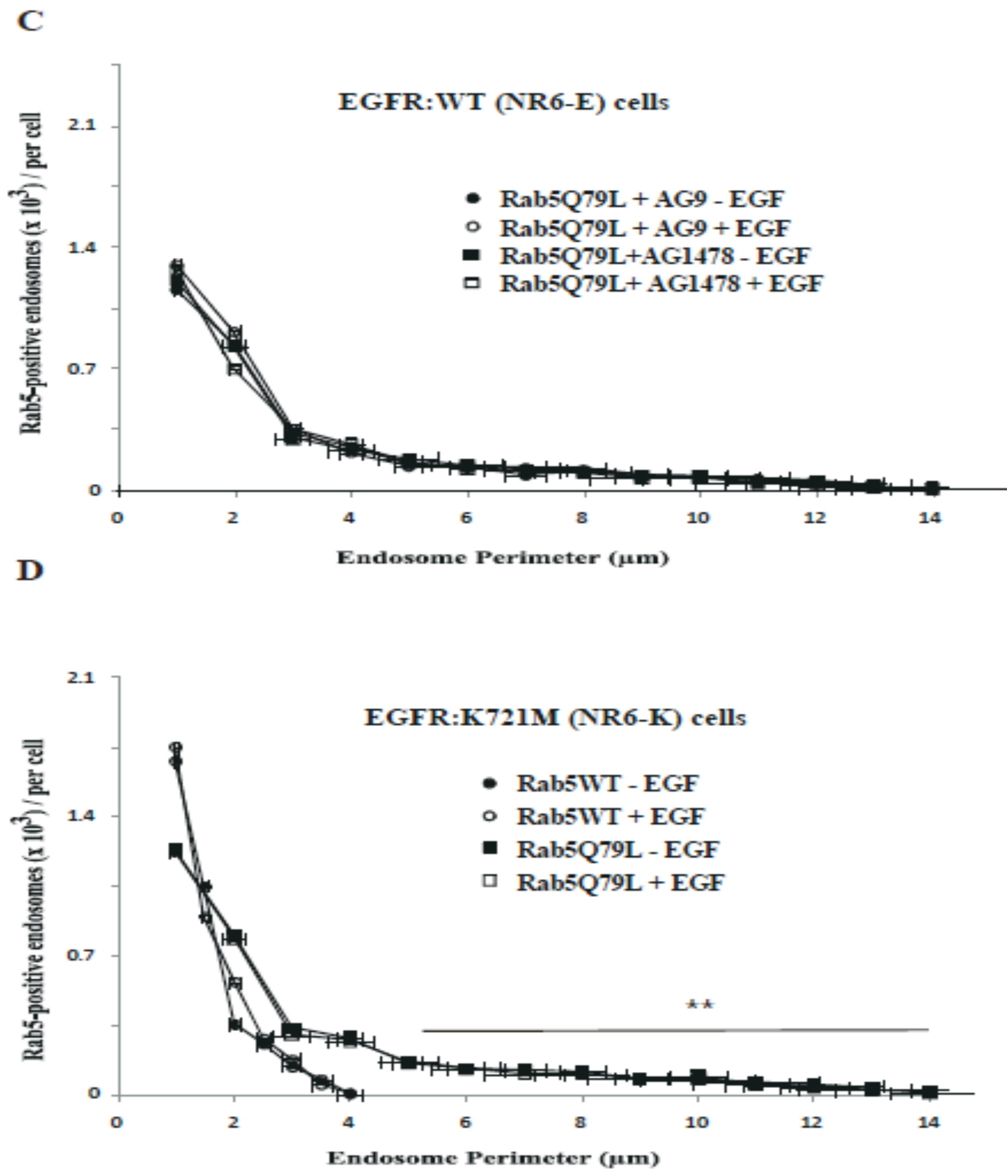
Suppl. Figure 1. Confocal immunofluorescence analysis of cells co-expressing EGF-receptor, Rab5: Q79L mutant in the presence of EGF-receptor tyrosine kinase inhibitor. NR6 cells expressing human EGF-receptor wild type (A-L) and catalytically inactive mutant (K721M) (M-R) were transfected with plasmids encoding GFP-Rab5: Q79L (A-R) and then incubated in the absence (A-C, G-I and M-O) or in the presence of EGF (D-F, J-L and P-R). Cells were also supplemented with 100 nM AG1478 inhibitor (G-L) and 100 nM AG9 (inactive analog) (A-F). 100 ng/ml EGF was bound to the cells at 4°C for 60 min. Cells were then washed with ice-cold PBS, incubated at 37°C for 8 min, washed three times with ice-cold PBS, fixed with 4% paraformaldehyde and then were permeabilized with 0.1% Triton X-100 prior to incubation with rabbit anti-EGF-receptor and Alexa564-labelled donkey anti-rabbit antibodies, respectively. Yellow color indicates areas of co-localization between the internalized EGF-receptor and the overexpressed GFP-Rab5: Q79L mutant (C, F, I, L, O and R). An inactive analog (AG9) was used as control. The optical sections viewed are 0.4 µm. Size bars, 10 µm.



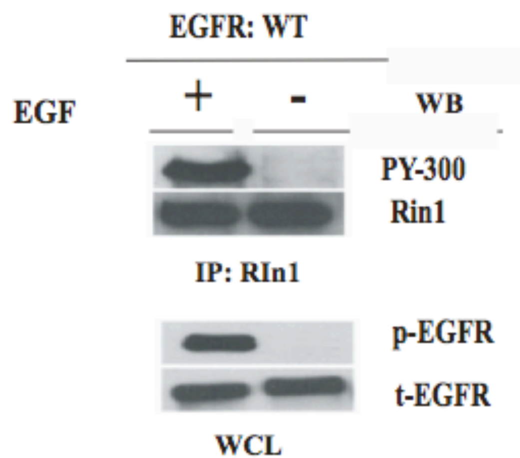
Suppl. Figure 2. Expression and tyrosine phosphorylation of EGF-receptor in cells expressing Rab5 proteins. NR6 cells expressing human EGF-receptor wild type (WT) and catalytically inactive mutant (K721M) were transfected with plasmids encoding GFP-Rab5: wild type (A) and GFP-Rab5:Q79L mutant (B) as indicate in the Figure. Cells were starved, stimulated or not for 8 min with EGF (100 ng/ml), and lysed in ice-old lysis buffer as described in Material and Methods. Cell lysates were then subjected to SDS-PAGE and analyzed by immunoblotting using anti-phospho (p) and anti-total (t)-EGF-receptor and anti-Rab5 antibodies. The experiment was repeated twice with similar results.


A
EGFR:WT (NR6-E) cells
Rab5WT – EGF
Rab5WT + EGF
Rab5WT + AG9 – EGF
Rab5WT + AG9 + EGF
Rab5-positive endosomes (x 10^3) / per cell
Endosome Perimeter (µm)
2.1
1.4
0.7
0
1
3
5
*


B
EGFR:WT (NR6-E) cells
Rab5WT + AG9 – EGF
Rab5WT +AG9 + EGF
Rab5WT + AG1478 – EGF
Rab5WT + AG1478 + EGF
Rab5-positive endosomes (x 10^3) / per cell
Endosome Perimeter (µm)
2.1
1.4
0.7
0
1
3
5
*



Suppl. Figure 3. Effect of AG1478 on the diameter of Rab5-positive endosomes. Perimeters of Rab5-positive endosomes from at least 200 cells were measured using the NIH Image J software from immunofluorescence images acquired using a Leica TCS SP2 confocal microscope in NR6 cells for each experimental condition. (A-B) Histograms of Rab5-positive endosomes/cell vs. the endosome size were determined in NR6-E cells expressing Rab5 wild type (WT) alone or containing either 100 nM AG9 or 100 nM AG1478 in the absence or in the presence of 100 ng/ml EGF. Asterisk (*) denotes an extended tail of Rab5: WT endosomes distribution in the presence of EGF. (C) Histograms of Rab5-positive endosomes vs. the endosome size were determined in NR6-E cells expressing Rab5: Q79L containing either 100 nM AG9 or 100 nM AG1478 in the absence or in the presence of 100 ng/ml EGF. (D) Histograms of Rab5-positive endosomes vs. the endosome size were determined in NR6-K cells expressing Rab5: Q79L in the absence or in the presence of 100 ng/ml EGF. Asterisks (**) denote an extended tail of Rab5: Q79L endosomes distribution in the presence of EGF. The data are presented as means ± SD of four independent experiments.



Suppl. Figure 4. Rin1 is not tyrosine phosphorylated by EGF. NR6 cells expressing human EGF-receptor wild type (WT) were transfected with plasmids encoding Rin1: wild type as indicated in the figure. Cells were serum-starved, stimulated or not for 8 min with EGF (100 ng/ml), lysed and then immunoprecipitated with anti-Rin1 antibodies as indicated in Material and Methods. The immunoprecipitates were then subjected to SDS-PAGE and analyzed by immunoblotting using anti-Rin1 and anti-phosphotyrosine (PY-300) antibodies, respectively. Whole cell lysates (WCL) were analyzed utilizing antibodies recognizing both total (t)- and phospho (p)-EGF receptor. The experiment was repeated twice with similar results.

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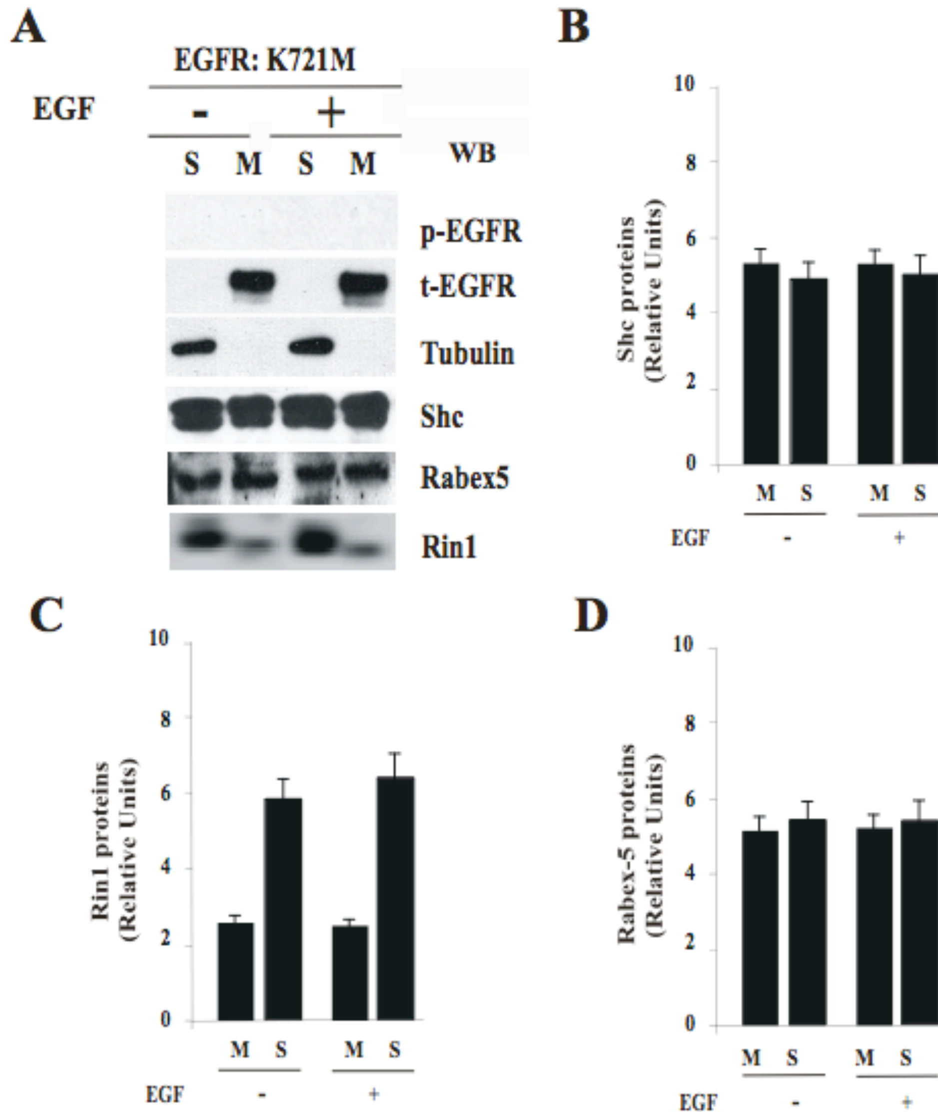
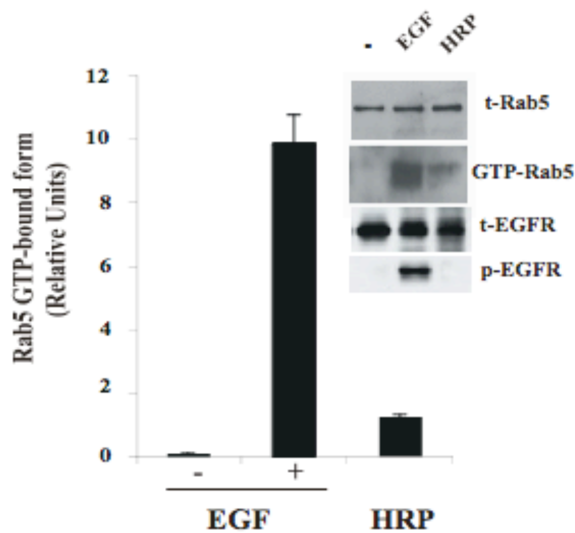


Suppl. Figure 5. Expression of EGF-receptor catalytically inactive mutant affects the recruitment of Rin1 to membrane upon stimulation of EGF. NR6 cells expressing EGF-receptor catalytically inactive mutant (K721M) were incubated in the absence or in the presence of 100 ng/ml EGF at 37°C for 8 minutes as indicated in Materials and Methods. Cells were then washed again using ice-cold PBS, homogenized and membrane fractions were prepared as described in Materials and Methods. Membrane [M] and cytosol [S] fractions were (A) treated with sample buffer and proteins were subject to SDS-PAGE, blotted to a nitrocellulose membrane, and antibodies specific to Shc (B), Rin1 (C), Rabex-5 (D), phospho(p)-EGF-receptor and total(t)-EGF-receptor, and tubulin (A) were used to visualize these proteins by Western blot analysis. Relative levels of these proteins were determined by densitometry. Data represents the mean ± SD of three independent experiments.



Suppl. Figure 6. Activation of Rab5 during fluid phase and receptor-mediated endocytosis. NR6 cells expressing EGF-receptor wild type were incubated in the absence or in the presence of 100 ng/ml EGF and 2 mg/ml HRP as indicated in the Figure. Each ligand was incubated with cells at 4°C for 60 min. Cells were then incubated at 37°C for 5 min. Subsequently, the cells were washed three times with ice-cold PBS, lysed and incubated with glutathione beads either in the presence of GST alone or GST-EEA1 at 4°C for 60 min. After incubation, the beads were washed and the presence of activated Rab5 was analyzed by Western blotting. The data are presented as means ± SD of four independent experiments.